

Vaccine Research Center

Immunology Core Laboratory



National Institute of Allergy and Infectious Diseases
National Institutes of Health

IMMUNOLOGY CORE LAB

Mission

- ❖ Develop, Optimize, and Validate Assays to Monitor Candidate Vaccine(s) Immunogenicity

Parameters

- ❖ Test frozen PBMCs
- ❖ Assays performed per GLP (Good Laboratory Practices: CFR Title21 Part 58)
 - PPE, SOPs, instrument and procedural controls, training documentation, documentation of equipment maintenance/QC/Calibration
 - ICH (International Conference on Harmonization: <http://www.ich.org>)
- ❖ Assays will be sensitive, quantitative, and reproducible
- ❖ Assays will measure a function
 - ICS and Elispot

IMMUNOLOGY CORE LAB

Long-Term Goals

- ❖ Addition of memory/naive, cytokine, and other functional markers (12-color)

- ❖ Increase automation of testing formats for expansion to Phase II/III trials

Optimization versus Validation

OPTIMIZATION

- ❖ Identifies critical junctures/conditions of an assay
- ❖ Tests each parameter
- ❖ Maximum antigen specific response
- ❖ Minimum background.
 - Reagents/Consumables
 - Incubation Times
 - Instrumentation
 - Analysis (Gating Strategy)



VALIDATION

- ❖ Confirms the optimization
- ❖ Day-to-day assay reproducibility
 - Inter and Intra assay variability
- ❖ Determine positivity criteria.



Optimization of ICS Assay Parameters

- Blood Collection
- Blood Processing/Freezing
- PBMC Thawing
- Pre-stimulation culture
- Stimulation
- Fix/Perm
- Staining
- Data collection/analysis

Pre-test Parameters
(Patient Visit)

Test Parameters
(Batch Immune
Assays)

Post-test Parameters
(End of clinical trial)

Optimization of ICS Assay Parameters

- **Blood Collection**

- Blood Processing/Freezing
- PBMC thawing
- Pre-stimulation culture
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- Staining
- Data collection/analysis

Parameters tested:

- Anticoagulants (Heparin, EDTA, ACD)
- CPT vs Ficoll-Paque™ Plus

Outcome measurements

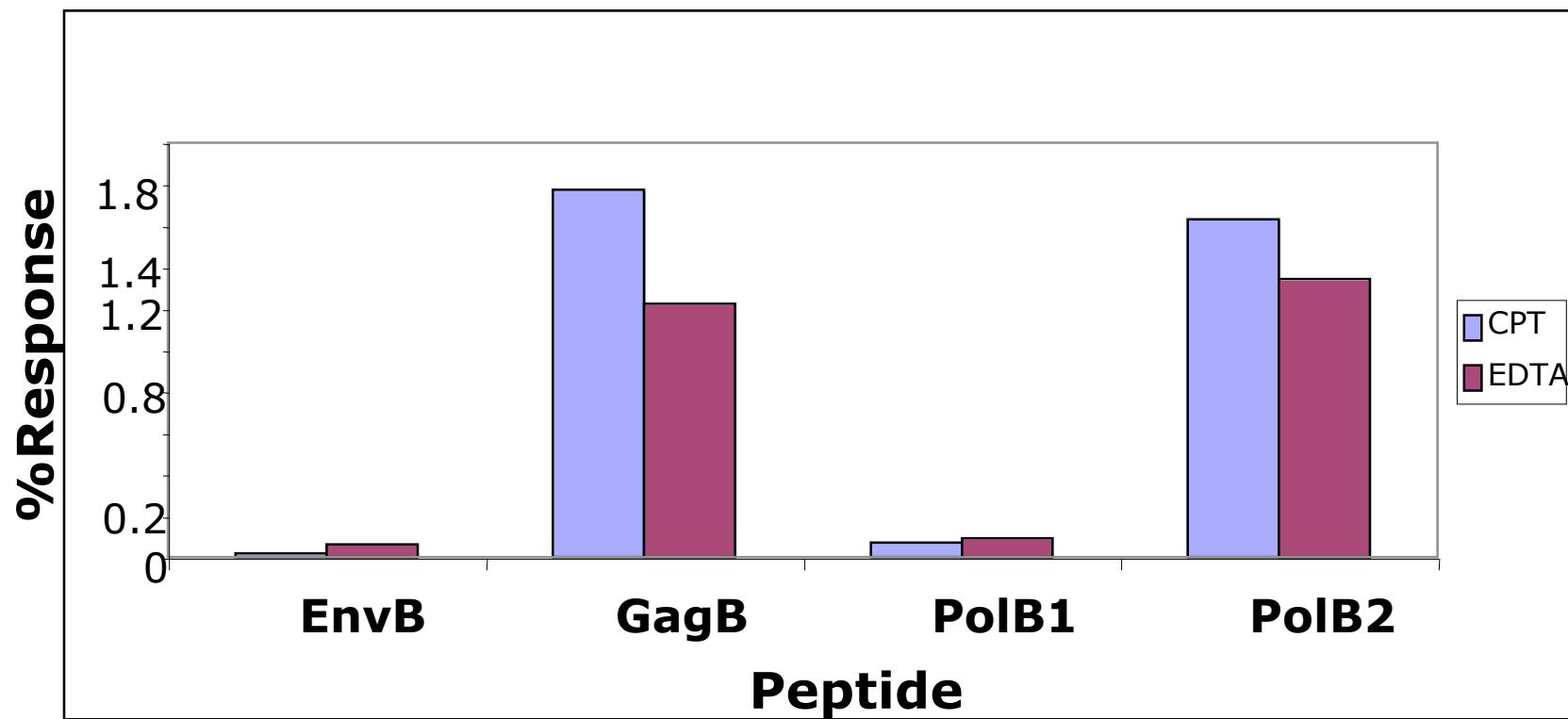
- Viability
- Yield (Recovery)
- Maximal functional response
- Minimal background

Conclusions

- EDTA
- CPT

Blood Collection

Background Corrected CD8 response for #57



Optimization of ICS Assay Parameters

- Blood Collection
- **Blood Processing/Freezing**
- PBMC thawing
- Pre-stimulation culture
- Stimulation
- Fix/Perm
- Staining
- Data collection/analysis

Parameters tested:

- Different cell separation/freezing protocols (different labs)

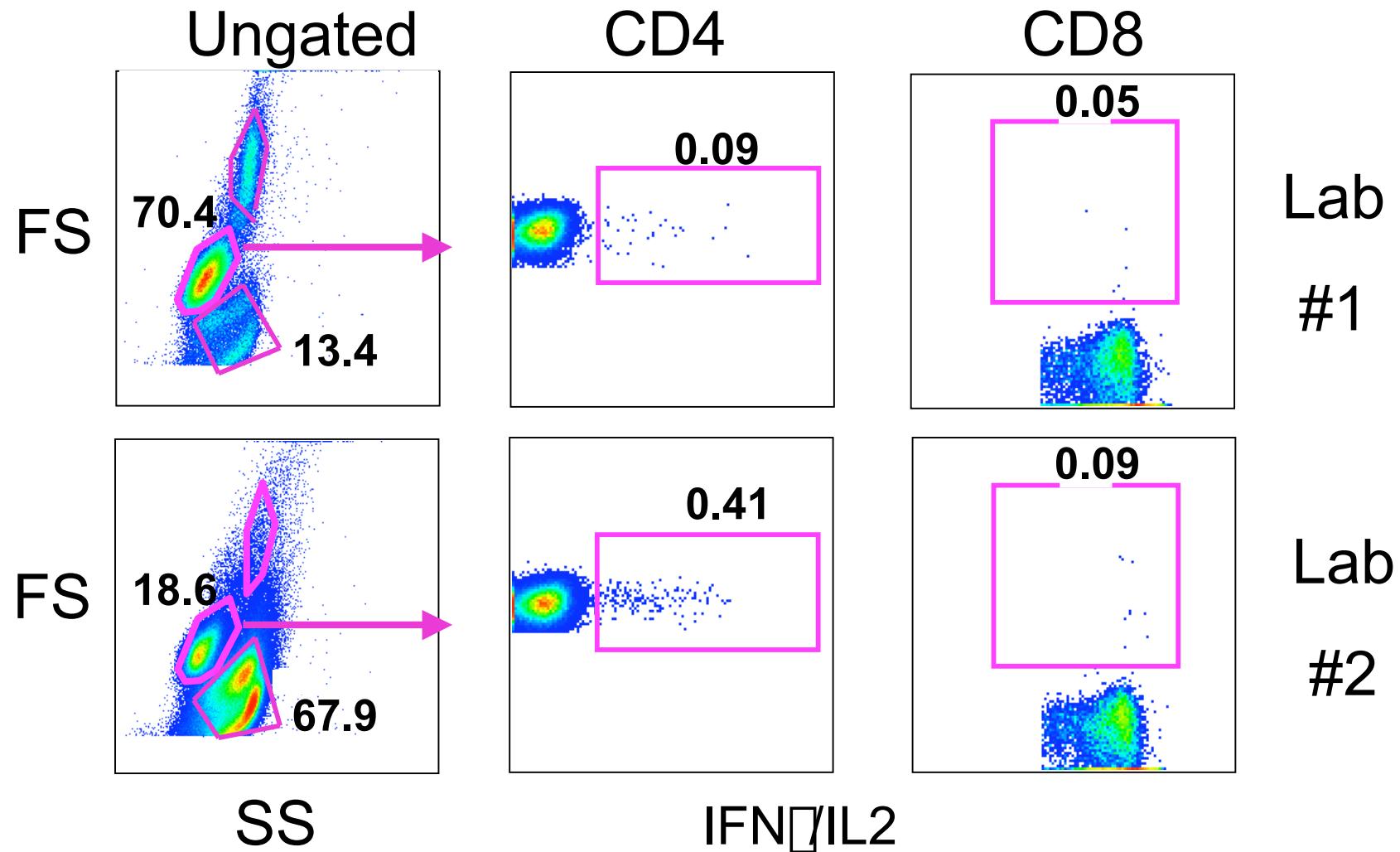
Outcome measurements

- Viability
- Yield
- Maximal functional response
- Minimal background

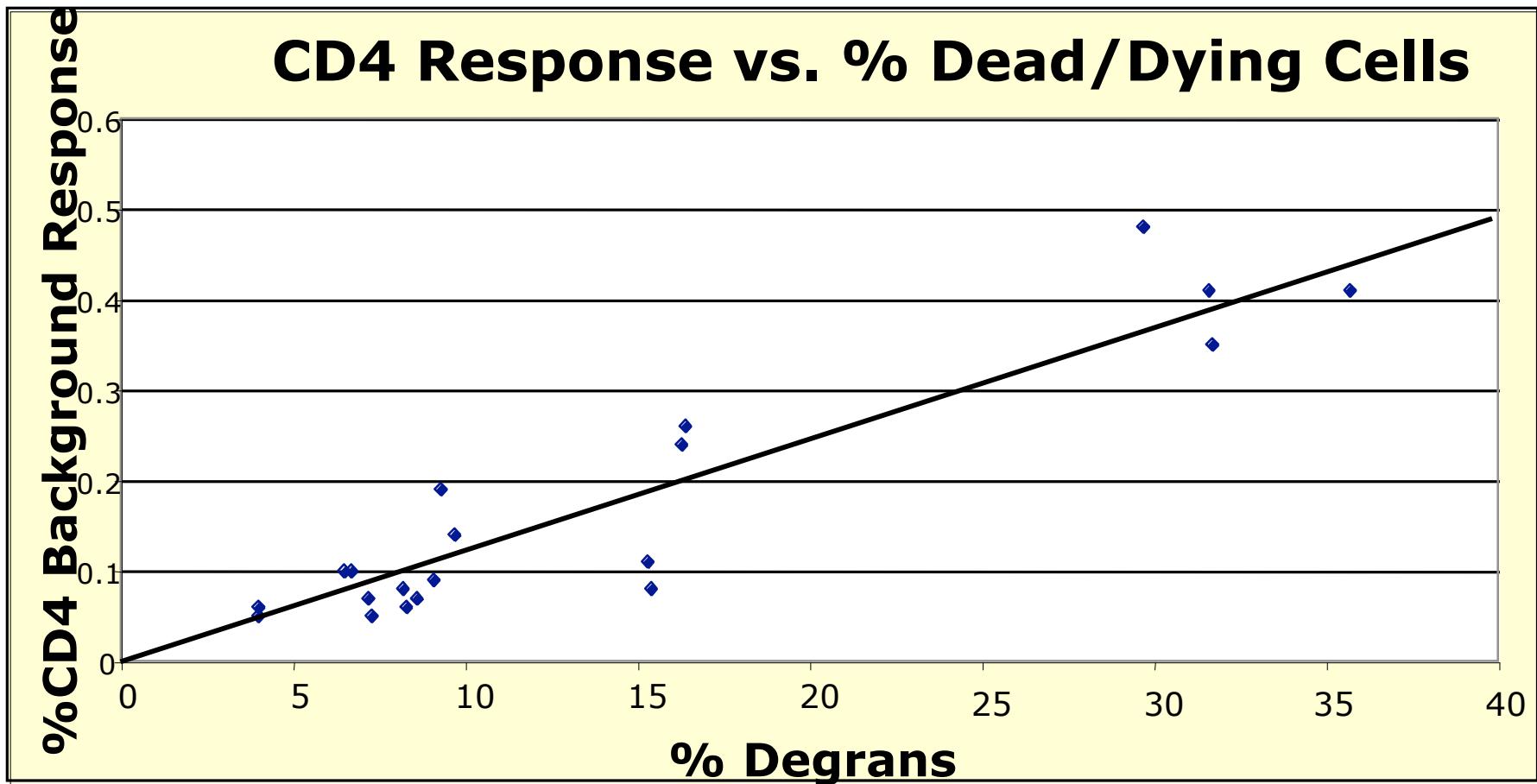
Conclusions

- Use our SOP
- Control rate freezer

PROCESSING: It's the Little Things.....



Processing



Cryopreservation methods were performed by another institution

Optimization of ICS Assay Parameters

- Blood Collection
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- Staining
- Data collection/analysis

Parameters tested:

- Different media thaw temperatures

Outcome measurements

- Viability
- Yield

Conclusions

- Thaw in prewarmed media

Thawing PBMCs

Conditions:

- 5×10^6 cells/vial.
- Thaw in 15 mL R10 at different temperatures
- Wash 2x, count

Sample	4C	25C	37C
299			
Recovery ($\times 10^6$)	1.8	3.4	3.5
Viability (%)	77	96	96
301			
Recovery ($\times 10^6$)	2.0	3.0	2.7
Viability (%)	74	98	99
363			
Recovery ($\times 10^6$)	1.8	3.2	3.0
Viability (%)	70	98	97

Optimization of ICS Assay Parameters

- Blood Collection
- Blood Processing/Freezing
- PBMC thawing
- **Pre-stimulation culture**
- Stimulation
- Fix/Perm
- Staining
- Data collection/analysis

Parameters tested:

- BFA vs. Monensin
- Time of Addition

Outcome measurements

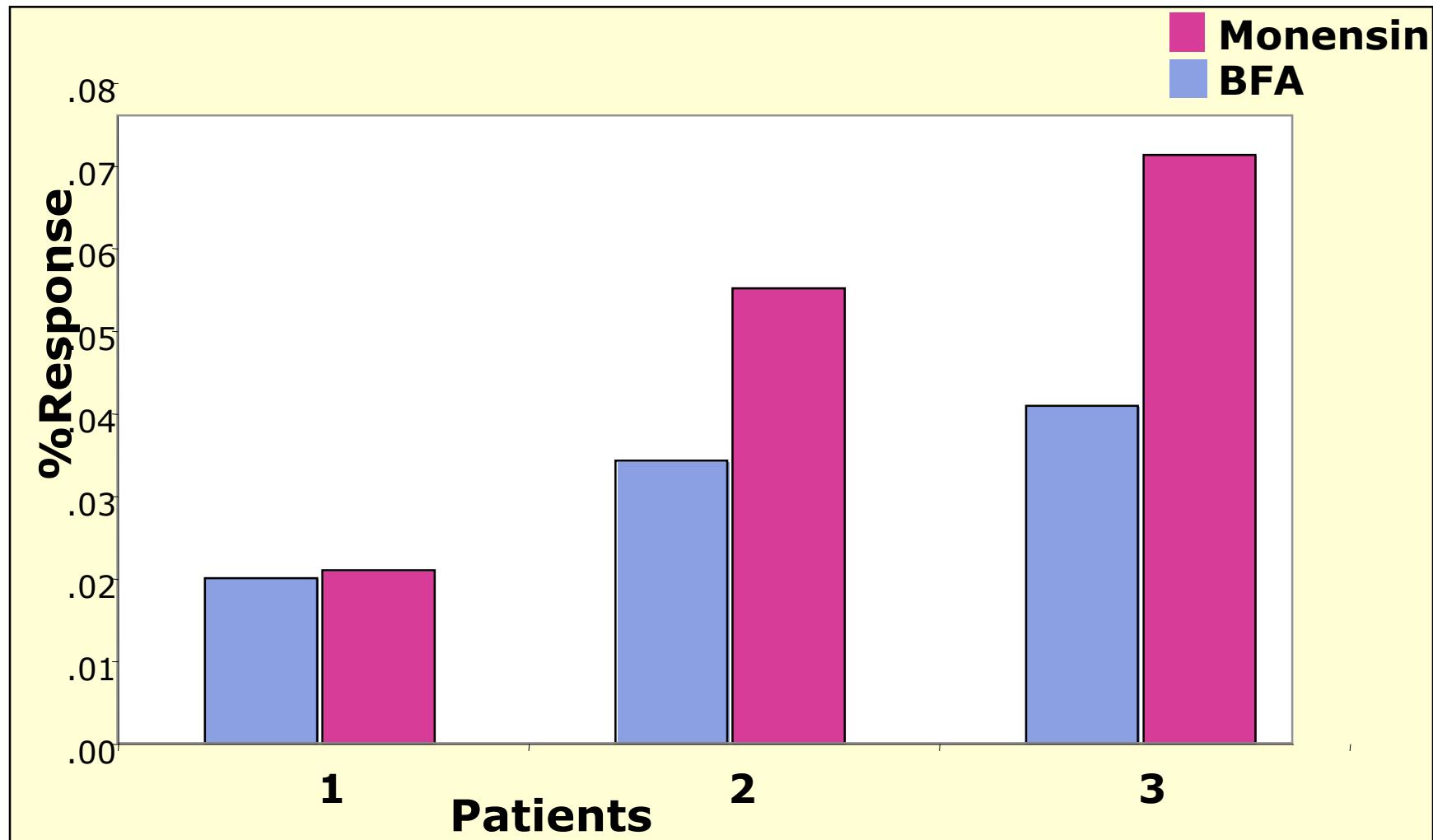
- Minimal Background
- Maximal Functional Response

Conclusions

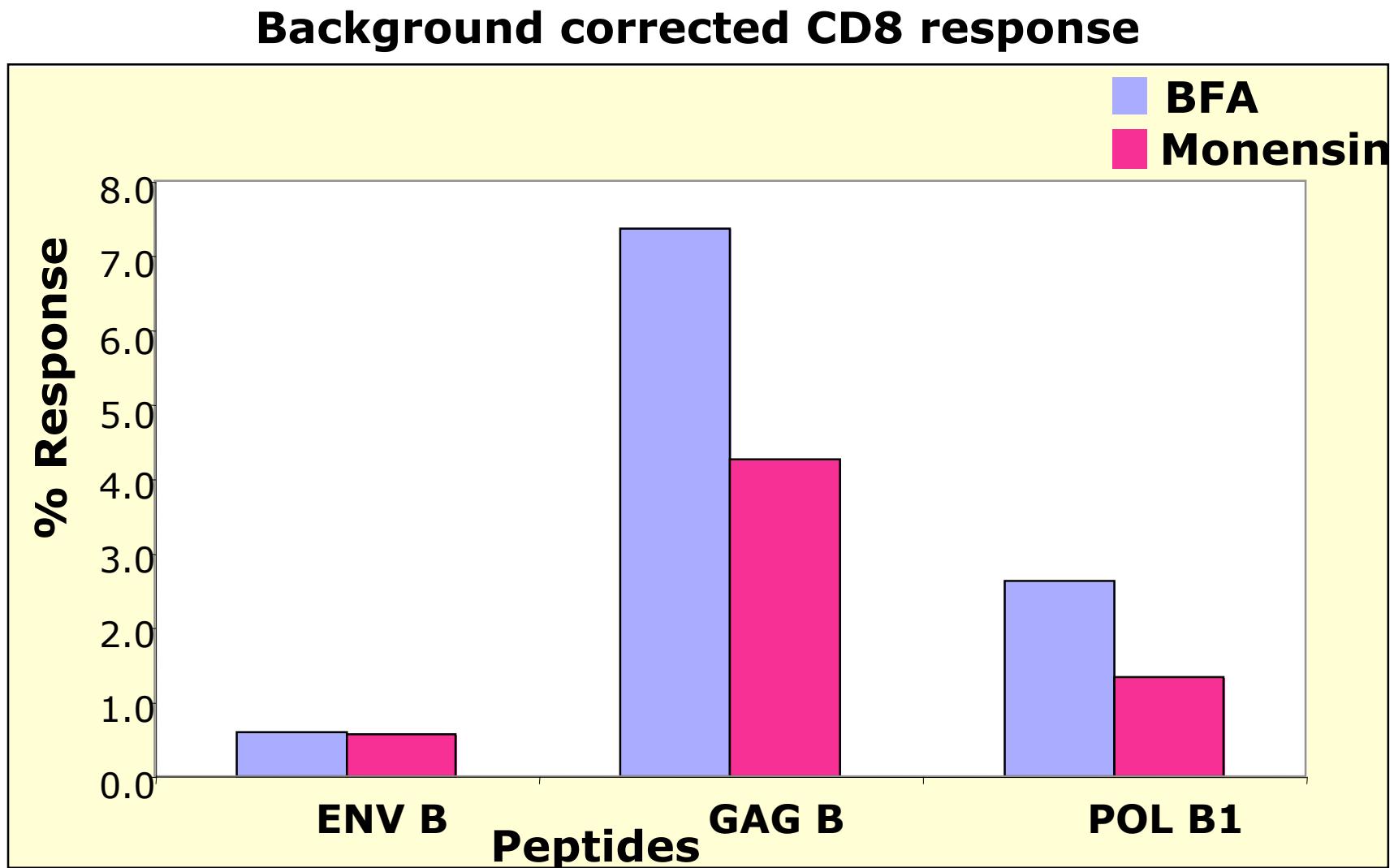
- Use BFA
- Add at 0 hr.

Prestim Culture: BFA vs. Monensin

CD4 Responses for Non-Stimulated Samples



Prestim Culture: BFA vs. MONENSIN



Optimization of ICS Assay Parameters

- Blood Collection
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- PBMC thawing
- Pre-stimulation culture
- **Stimulation**
- Fix/Perm
- Staining
- Data collection/analysis

Parameters tested:(6 total)

- 6 Hr. vs. Overnight Rest
- 6 Hr. vs. Overnight Stim
- Costimulation (28/49d)

Outcome measurements

- Yield/Viability Post Rest
- Minimal Background
- Maximal Functional Response

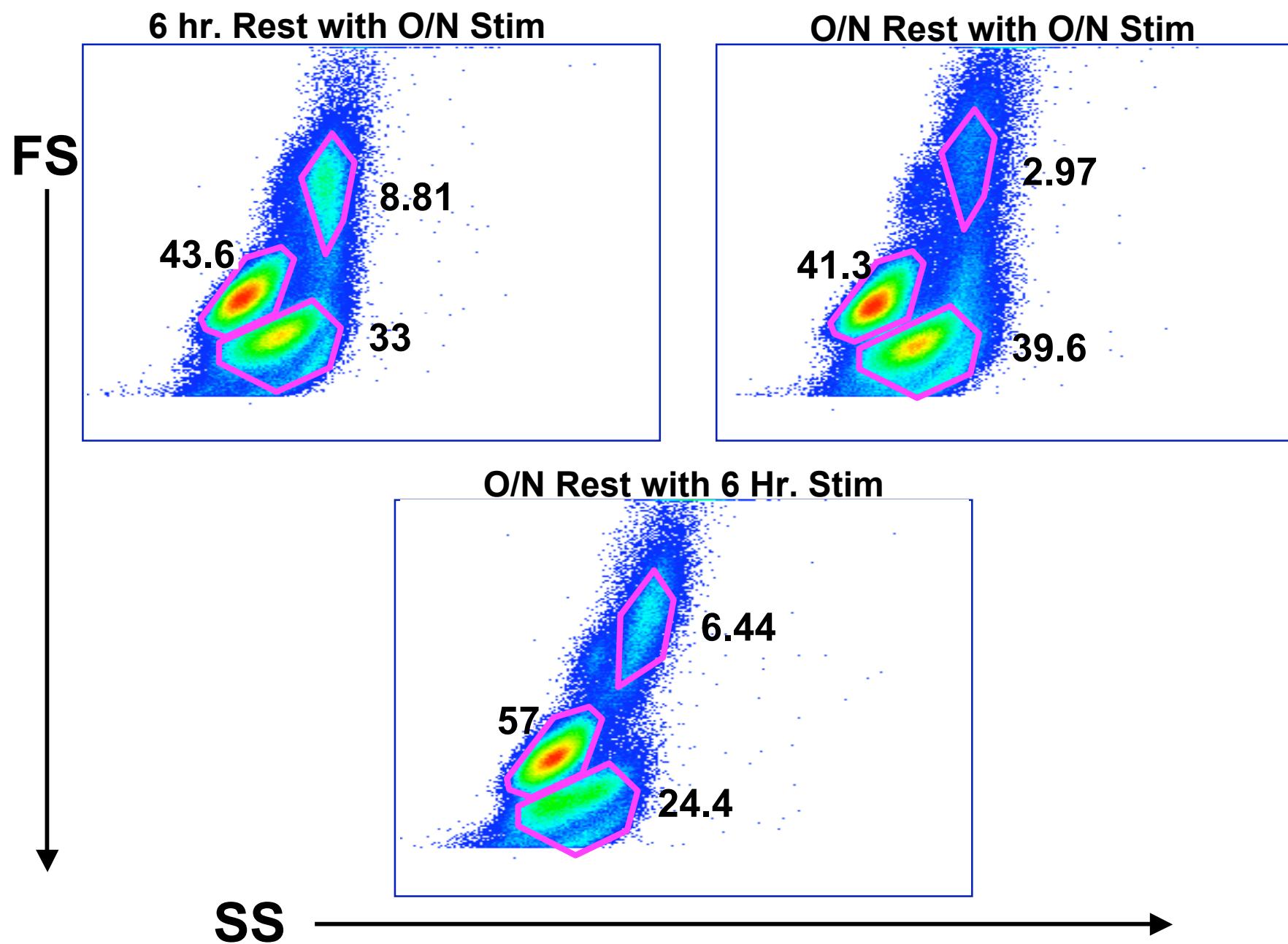
Conclusions

- Use Costimulation
- Stimulate 6 hrs.
- Rest overnight

Stimulation

Results

- Costimulation increased CD4 and CD8 responses for 6 hour stimulation
- 6 Hr stimulation increased CD4 and CD8 responses
- Overnight rest reduced background and improved CD8 responses
- Overnight rest (prior to overnight stims) further increased responses slightly, but at a cost of altered cell populations



Optimization of ICS Assay Parameters

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- Stimulation
- **Fix/Perm**
- Staining
- Data collection/analysis

Kits tested:

- BD FACSlyse with Tween20
- Caltag
- PharMingen

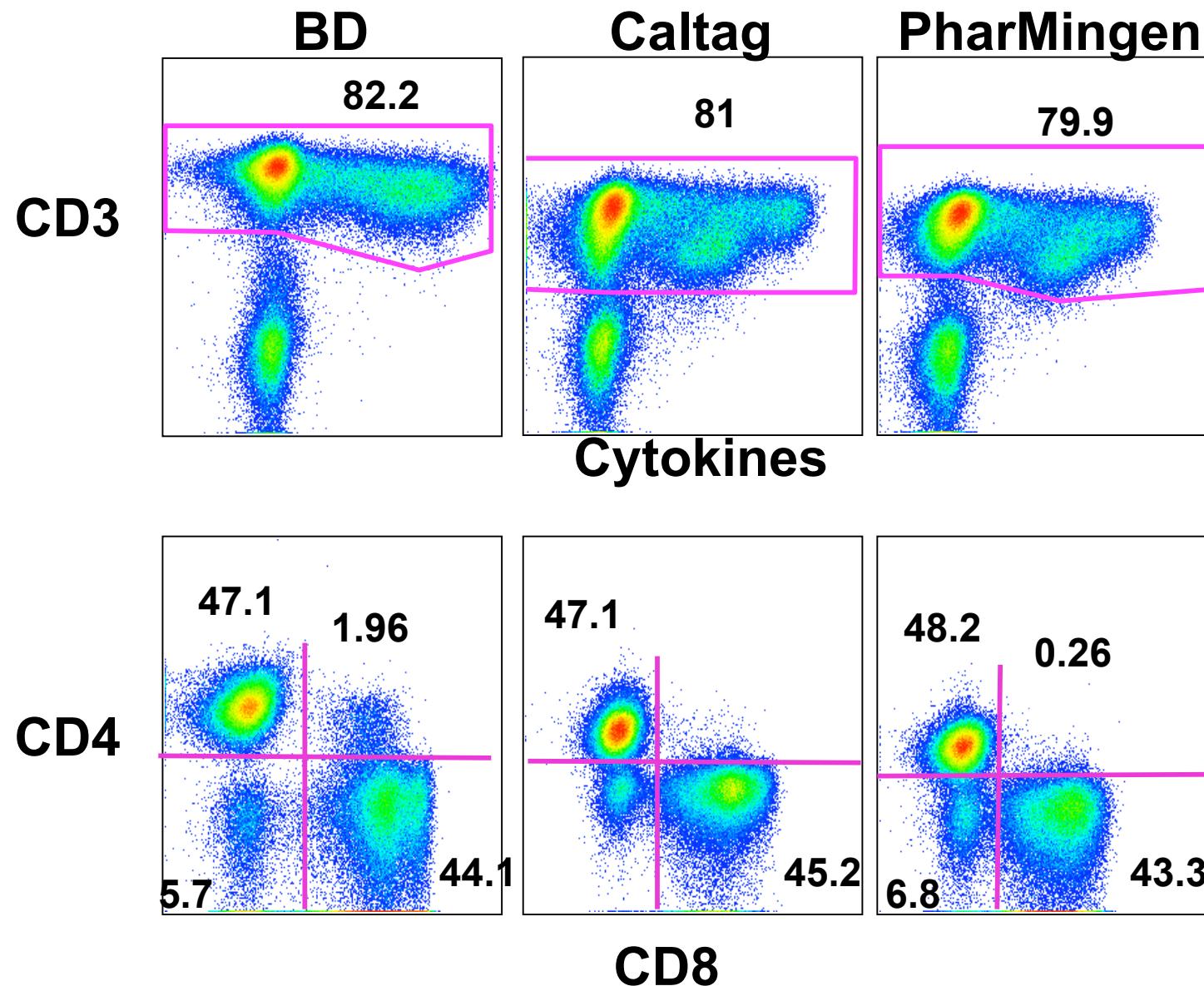
Outcome measurements

- Stain Intensity
- Positive/negative separation
- Minimal Background
- Maximal Functional Response

Conclusions

- Use BD FACSlyse with Tween20

Fixation/Permeabilization Methods



Optimization of ICS Assay Parameters

- Blood Collection
- Blood Processing/Freezing
- PBMC thawing
- Pre-stimulation culture
- Stimulation
- Fix/Perm
- **Staining**
- Data collection/analysis

Parameters to consider:

- Conjugation of antibody to fluorochrome
- Custom-made monoclonals
- Titrate antibodies
- Which cytokines?

Outcome measurements

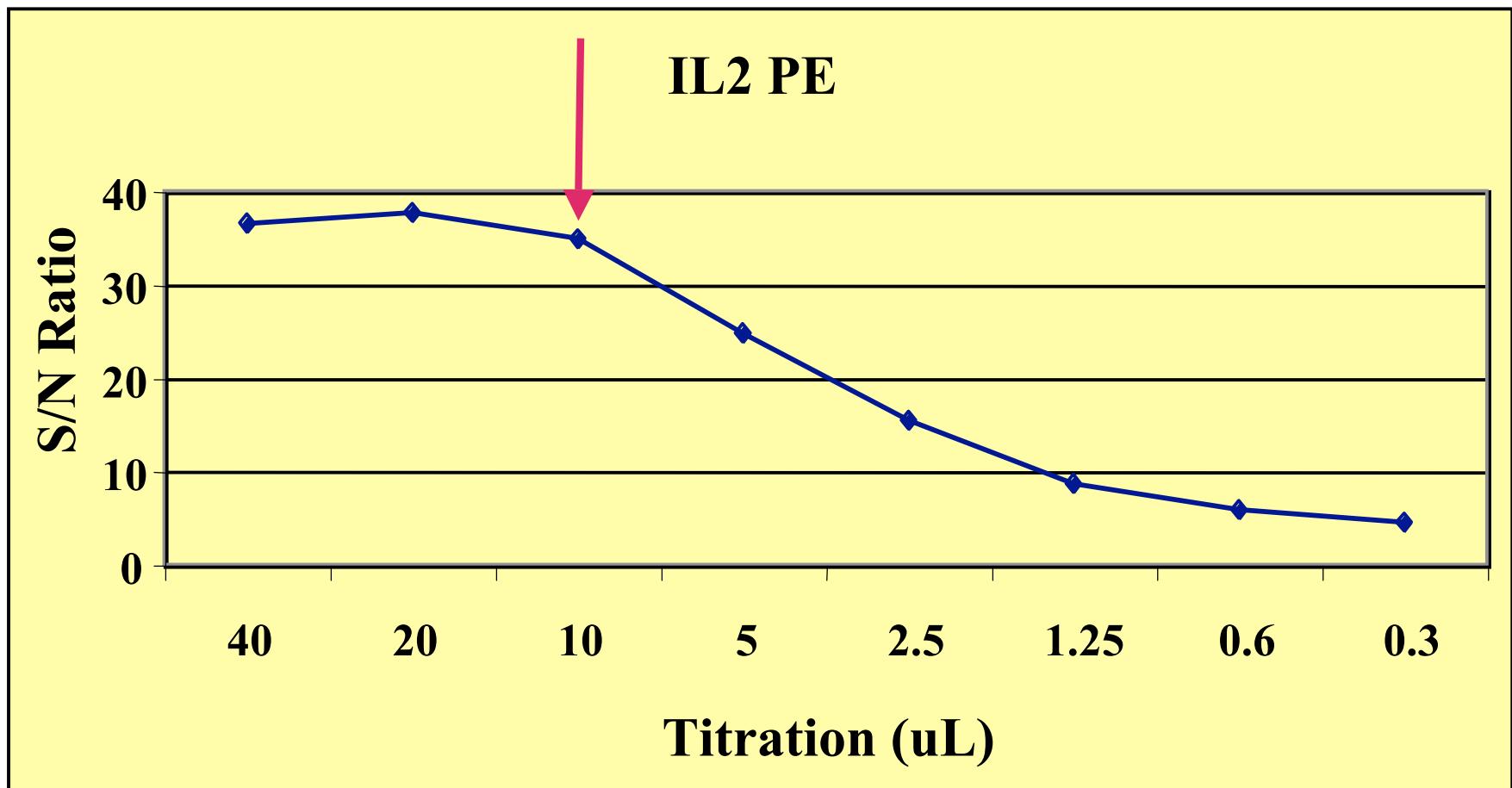
- S/N
- Minimal Background

Conclusions

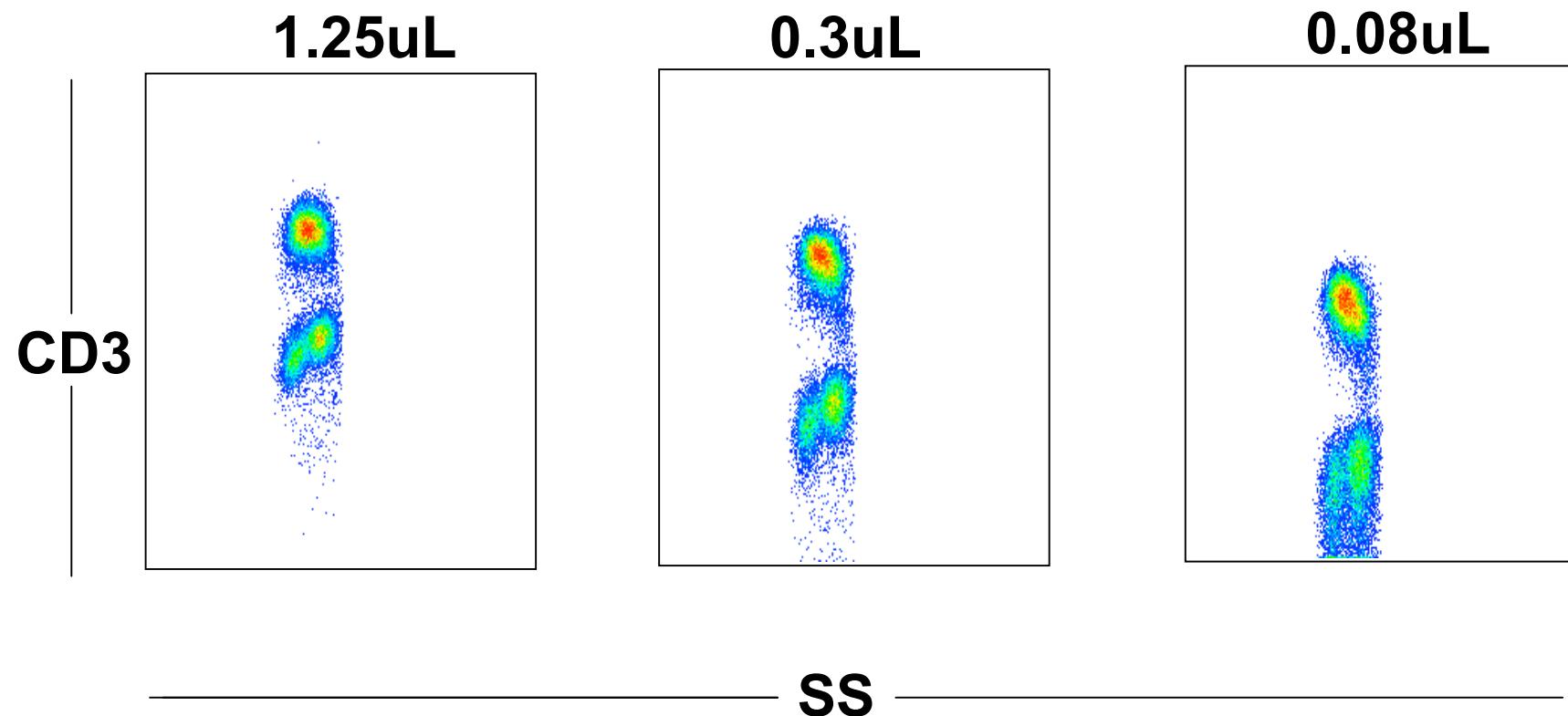
- Purchase custom-made monoclonals in bulk
- Stain for IFN γ and IL2 using same fluorescent marker

Staining: Antibody Titration

Titer of 10 uL yields optimal separation of positive and negative populations



Staining: Antibody Titration



Staining: Which Cytokines?

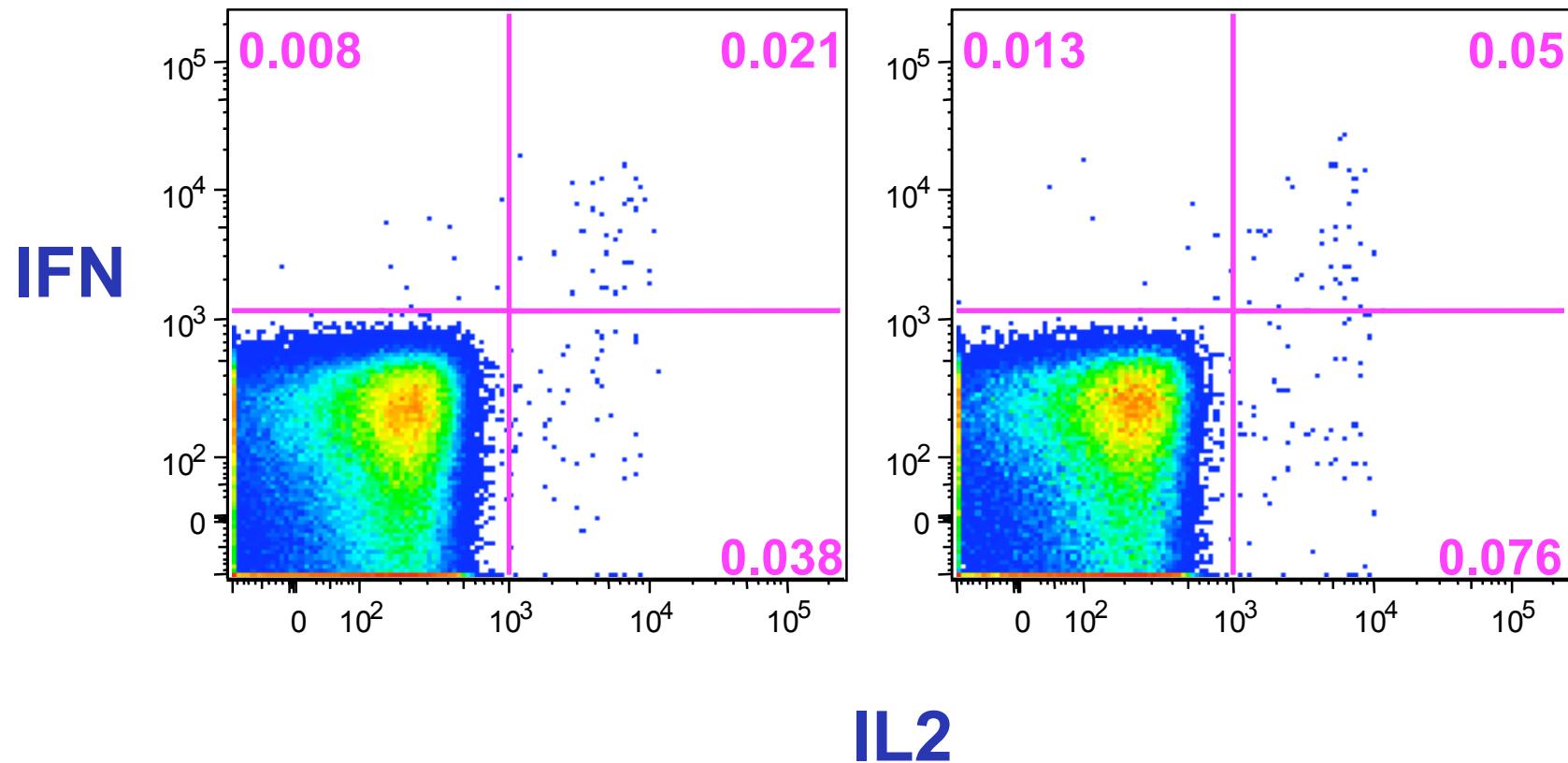
Previous studies (De Rosa & Roederer) showed that primary immune responses are comprised of complex cytokine profiles...For example, many cells make IL2 but not IFN β . In fact, IFN β is subdominant.

At this time, it is not known which T cell response is most predictive of a successful vaccine.

Initially, it is not necessary to measure various cytokines independently: We are only interested in which cells make **any cytokine**, not which make particular cytokines. Later, we can identify the specific response.

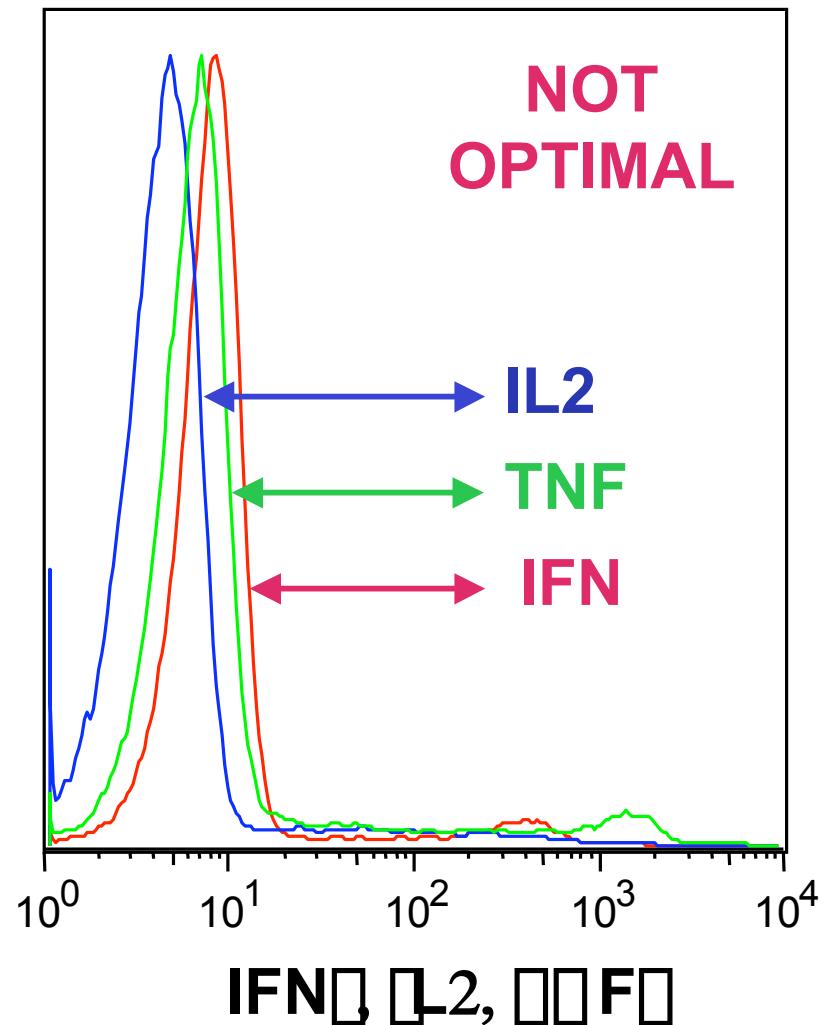
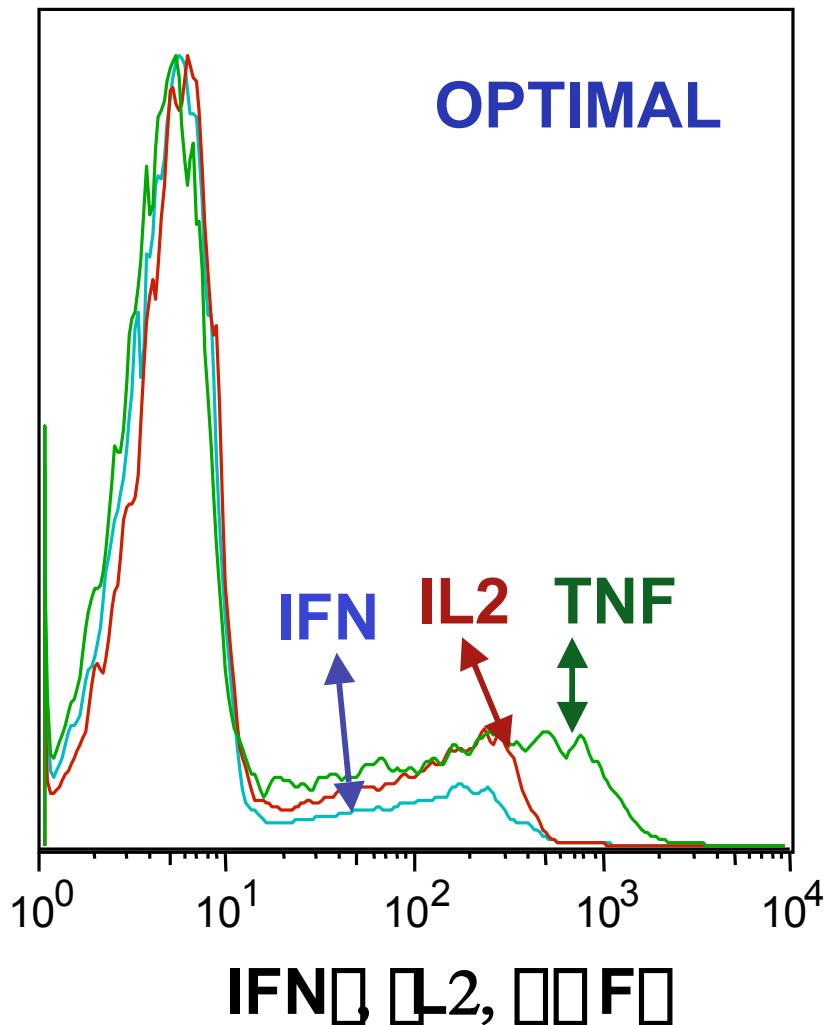
IFN \square is Not Enough!

2 subjects from VRC004 (DNA vaccine)

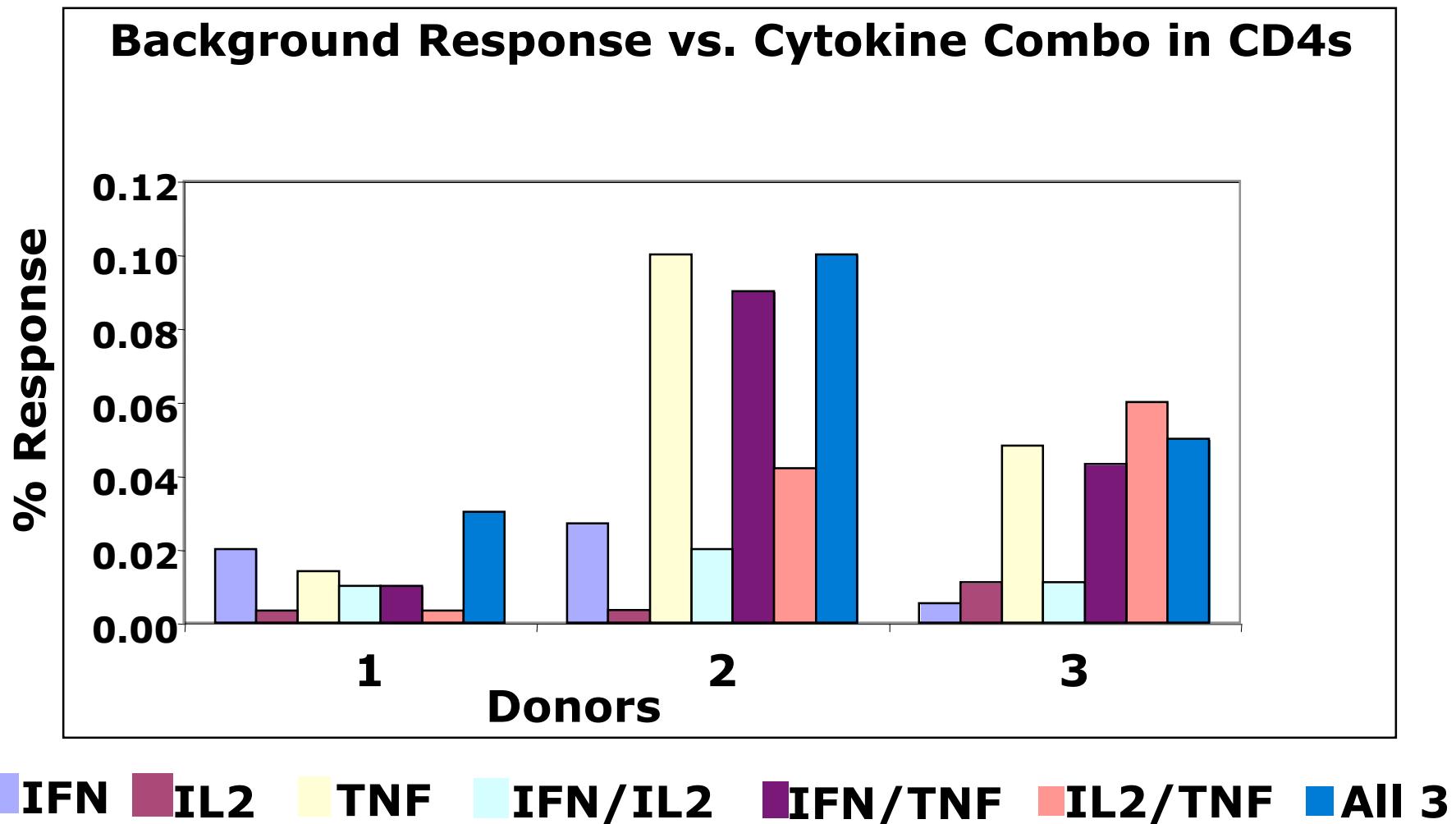


Gating: singlets, live, lymphocytes, CD14-, CD19-, CD3+, CD4+, CD8-

Staining: Cytokine Overlay



Staining: TNF Woes



Optimization of ICS Assay Parameters

- Blood Collection
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- Stimulation
- Fix/Perm
- Staining
- **Data collection/analysis**

Parameters to consider:

- Classic vs. Inclusive Gating
- High vs. Low Gating

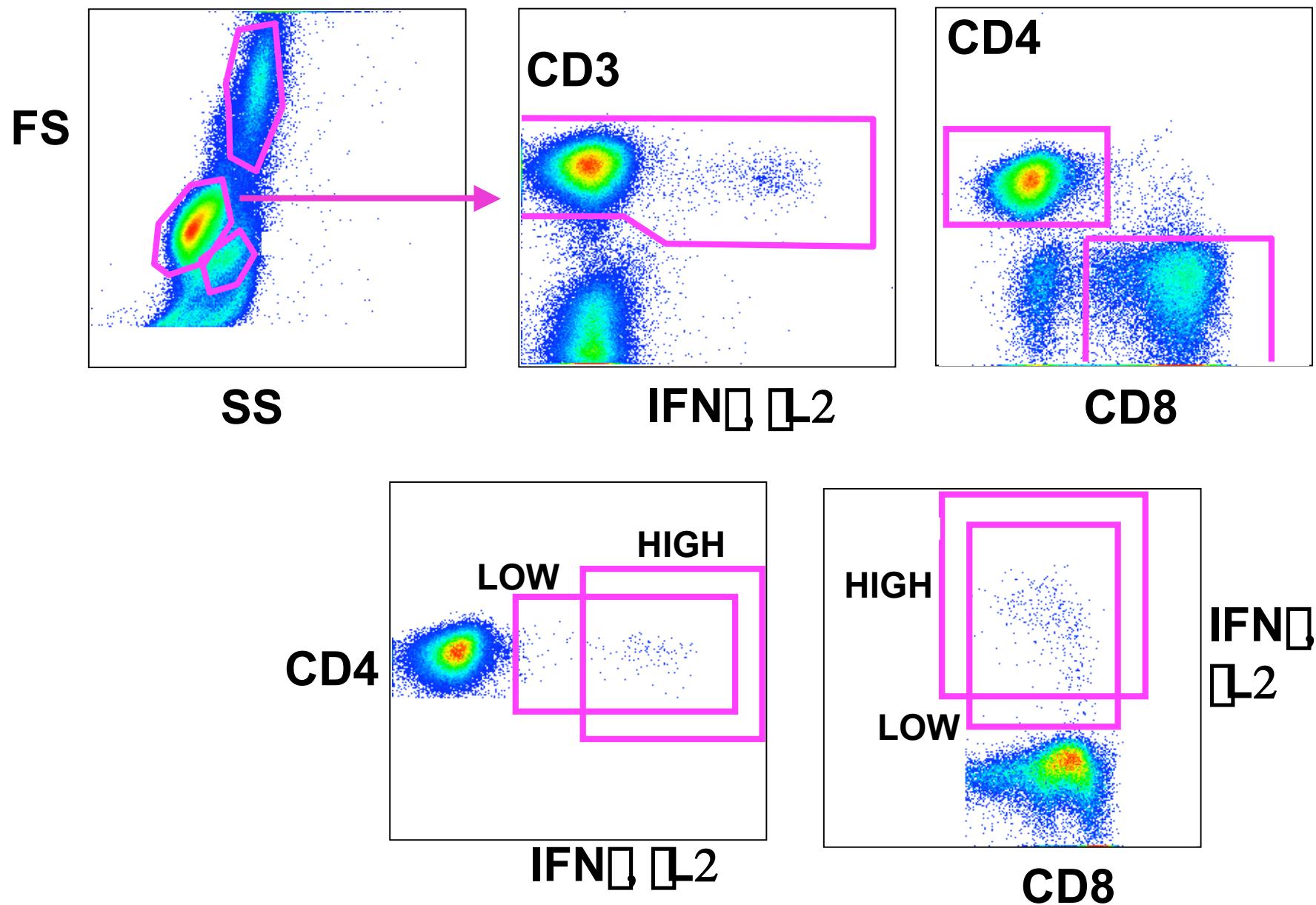
Outcome measurements

- Backgate cytokine responses

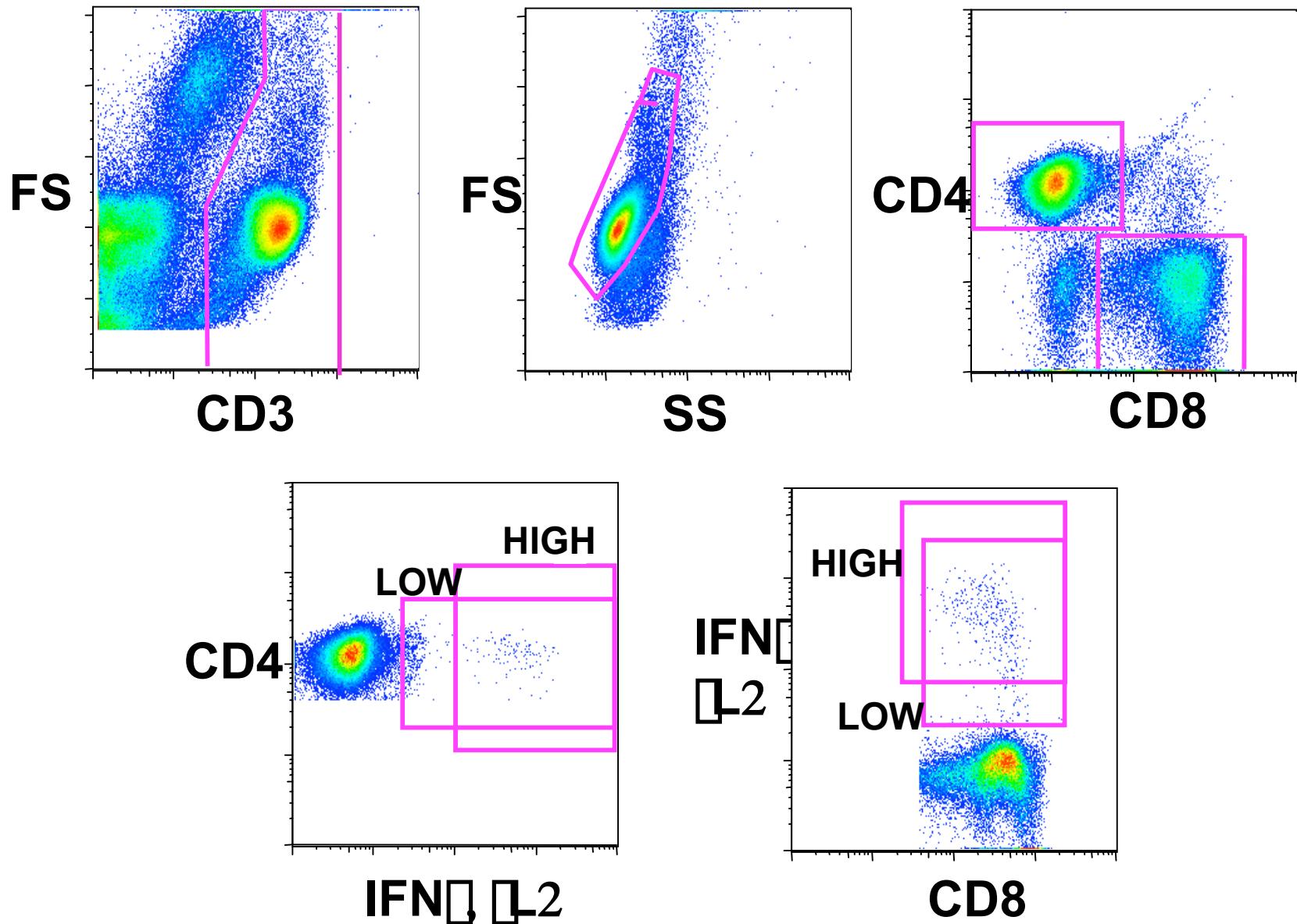
Conclusions

- Inclusive Gating
- High Gating

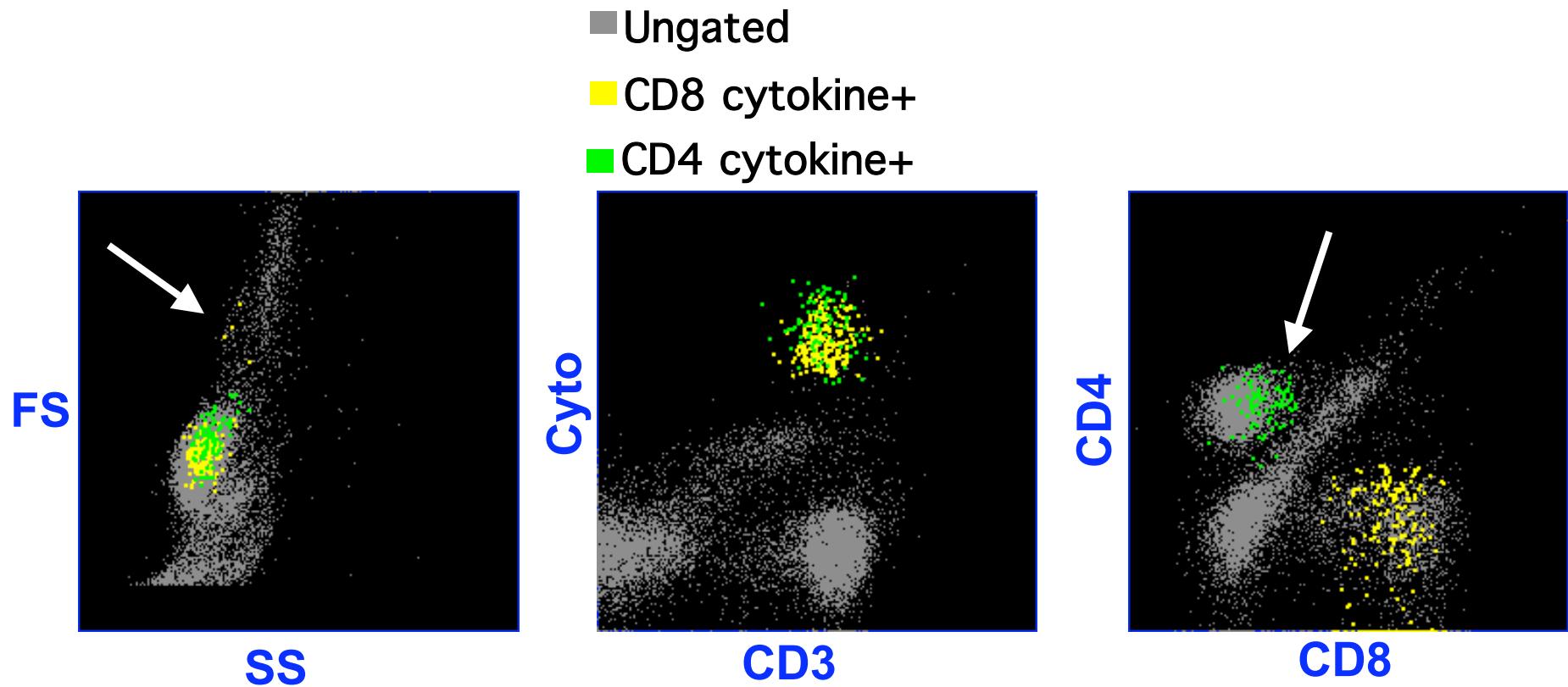
Data Analysis using Classic



Data Analysis using Inclusive



Data Analysis: Backgate Cytokine Responses



- **Observances**
 - Where are cytokine producing cells located?
- **Decision**
 - Use broader gates to capture larger lymphs and all CD4 cytokine producers

Assay Validation Based on GLP

(CFR Title21 Part 58)

- Phase III study methods must be validated.
 - Validate as early in the licensure path as possible.
- Validation Parameters
 - Sensitivity - (true positives/true positives + false negatives)
 - Specificity - (true negatives/ true negatives + false positives)
 - Accuracy
 - Precision- (Intra and Inter assay variablity)
 - Ruggedness
 - Robustness
- VRC Validation: 35 HIV+ and 35 HIV- donors
 - Diversity of donors
 - Repeat testing of donors

Assay Validation: Template Example

Run #	Analyst #1								Analyst #2							
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	Donor 7	Donor 8
1	1	1	1	2	2	2	3	3	3	1	2	3	4	5	6	7
2	1	1	1	4	5	1	8	9	2	2	2	6	7	8	10	11
3	1	2	9	10	1	12	13	14	11	12	13	14	15	15	16	17
4	1	1	16	17	18	19	18	19	20	21	22	23	20	21	22	23
5	24	25	26	27	24	25	26	27	1	1	28	29	30	31	28	29
6	32	33	34	35	30	31	32	33	1	1	36	37	38	39	34	35

Plan Includes:

- 2 HIV+ donors tested in triplicate over 2 days and 2 analysts
- 3 HIV+ donors tested in triplicate within same day
- 1 HIV+ and 1 HIV- tested in every run via different analysts
- 2 HIV+ donors tested over 3 separate days/analysts
- HIV+ and - donors intermixed and blinded to analysts

GLP Assay/Validation: Other Considerations

- **Reagents**
 - Define critical analytical reagents that may impact results.
 - Purchase in bulk to prevent multiple lot changes.
 - Lot change must be parallel tested
- **Instrumentation**
 - Include all instruments (3 caliburs)
 - Validate system software
 - Vendor
 - Contractor
 - Deviations require equivalency testing
 - Perform QC each day of testing
 - Maintain maintenance logs
 - periodic review
 - Audit trail for all samples

GLP Assay/Validation: Other Considerations

- **Quality Control**
 - Positive and negative controls for each run
 - Establish control range
 - Chart and review
- **Deviations in Assay Procedure**
 - Document all deviations
 - Evaluate all deviations
 - If no impact, proceed
 - If deviation enhances procedure, restart validation
 - If it can't be determined whether there was an impact or the impact is negative, discard results and repeat analysis

GLP Assay/Validation: Other Considerations

- **Data Documentation**
 - 10 year retention, minimum
 - Secondary review
 - Laboratory Information System (LIMS)
 - Sample chain of custody
 - Bar Coding reduces paperwork!!!
 - Freezer Inventory
 - Data Storage
 - Donors blinded to clinical/laboratory staff
 - Need to know only
- **Personnel**
 - Orientation
 - Safety
 - Read and understand SOP
 - Training
 - Observe
 - Perform

Miscellaneous Considerations

- EB/AO vs. Trypan blue
 - Green is live
 - Red/Orange is dead
 - Does not stain RBC
 - Need fluorescent microscope
- Addition of DNase
 - Prevention of cell clumping
- RPMI Manufacturer Comparison
 - Use GLP certified companies
- Standardization of Calibur Instrumentation
 - Standardize population locations for easier gating in bulk
- Compensation
 - On line vs. Off line

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